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Abolition of Posttranscriptional Regulation of Nitrate Reductase Partially Prevents the Decrease in Leaf NO_3^- Reduction when Photosynthesis Is Inhibited by CO_2 Deprivation, but Not in Darkness

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The activity of nitrate reductase (NR) in leaves is regulated by light and photosynthesis at transcriptional and posttranscriptional levels. To understand the physiological role of these controls, we have investigated the effects of light and CO_2 on in vivo NO_3^- reduction in transgenic plants of *Nicotiana plumbaginifolia* lacking either transcriptional regulation alone or transcriptional and posttranscriptional regulation of NR. The abolition of both levels of NR regulation did not modify the light/dark changes in exogenous $^{15}NO_3^-$ reduction in either intact plants or detached leaves. The same result was obtained for ^{15}N incorporation into free amino acids in leaves after $^{15}NO_3^-$ was supplied to the roots, and for reduction of endogenous NO_3^- after transfer of the plants to an N-deprived solution. In the light, however, deregulation of NR at the posttranscriptional level partially prevented the inhibition of leaf $^{15}NO_3^-$ reduction resulting from the removal of CO_2 from the atmosphere. We concluded from these observations that in our conditions deregulation of NR in the transformants investigated had little impact on the adverse effect of darkness on leaf NO_3^- reduction, and that posttranscriptional regulation of NR is one of the mechanisms responsible for the short-term coupling between photosynthesis and leaf NO_3^- reduction in the light.

In many species of higher plants most organic N is derived from the assimilation of NO_3^- in the leaves (Beevers and Hageman, 1980; Gojon et al., 1994). The first enzymatic step of this assimilation, reduction of NO_3^- to NO_2^- by cytosolic NR, is considered to be the rate-limiting reaction, as well as the main regulatory point of the reduction of NO_3^- to NH_4^+ (Beevers and Hageman, 1980; Crawford, 1995). Light and sugars are among the important factors involved in the control of NO_3^- reduction in leaves, which is strongly inhibited in the absence of photosynthesis (Beevers and Hageman, 1980; Abrol et al., 1983; Kaiser and Förster, 1989; Delhon et al., 1995a, 1996a). The causes of the adverse effect of darkness on leaf NO_3^- reduction are still under debate (Delhon et al., 1996a), and several hypotheses have been proposed: (a) restriction of NO_3^- supply due to the inhibition of translocation from the roots (Rufty et al., 1984; Delhon et al., 1995a, 1995b), (b) limitation by the availability of reducing power (NADH for most NR isoforms) resulting from lowered generation by glycolysis and increased competition with mitochondrial respiration (Abrol et al., 1983), and (c) decreased NR activity (Beevers and Hageman, 1980; Remmler and Campbell, 1986).

Recently, major advances have resulted from the demonstration that NR is controlled by light at both transcriptional and posttranscriptional levels (Cheng et al., 1992; Vincentz et al., 1993; Huber et al., 1994; Kaiser and Huber, 1994a; Sivasankar and Oaks, 1996). The expression of the NR gene (*NIA*) in leaves is stimulated by light and by sugar supply (Cheng et al., 1992; Vincentz et al., 1993; Sivasankar and Oaks, 1996). However, the significance of this transcriptional control is questioned, since its suppression in transgenic *Nicotiana plumbaginifolia* plants (C1 transformant) does not modify the effect of light on leaf NR activity (Vincentz et al., 1993). Rather, the observations that light/dark transition or CO_2 deprivation are associated with both rapid inactivation and specific degradation of NR (Remmler and Campbell, 1986; Kaiser and Brendle-Behnisch, 1991; Huber et al., 1992; De Cires et al., 1993; Vincentz et al., 1993) led to the demonstration that the enzyme is strongly regulated at a posttranscriptional level (Huber et al., 1994; Kaiser and Huber, 1994a; Lillo, 1994a).

The posttranslational control of NR responsible for its reversible inactivation is based on an original two-step

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Abbreviations: ΔNR, nitrate reductase with a N-terminal deletion of 56 amino acids; NR, nitrate reductase; NRA, NR activity; NRP, NR protein; PGA, P-glycerate.

mechanism initiated by the ATP-dependent phosphorylation of a Ser residue (Huber et al., 1992; Kaiser and Huber, 1994a; Bachmann et al., 1996b; Moorhead et al., 1996). Phosphorylation alone has no effect on the activity of the enzyme, but allows its recognition by an NR inhibitor protein, identified as a mixture of 14-3-3 proteins (Bachmann et al., 1996a; Moorhead et al., 1996) that apparently bind to the phospho-NR in the presence of Mg^{2+} or Ca^{2+}, resulting in its inactivation (Glaab and Kaiser, 1995; MacKintosh et al., 1995). Reactivation of NR is initiated by dephosphorylation or by dissociation of the NR inhibitor protein (Kaiser and Huber, 1994a, 1994b).

Rapid inactivation of the leaf NR (50–90% inhibition of NRA after 30 min) has been shown to occur in various conditions in which photosynthesis is prevented, such as light/dark transition (Huber et al., 1992; De Cires et al., 1993), CO_2 removal from the atmosphere (Kaiser and Förster, 1989; Kaiser and Brendle-Behnisch, 1991), or stomatal closure in response to drought (Kaiser and Förster, 1989). Accordingly, hexoses or hexose-phosphates were identified as important metabolic effectors involved in the protection of NR against inactivation in vitro and in vivo (De Cires et al., 1993; Bachmann et al., 1995).

Until now, most of the data obtained at the biochemical level agree with the idea that posttranslational control of NR is a major regulatory mechanism governing amino acid synthesis in the leaves as a function of photosynthate production (Kaiser and Huber, 1994a). Unfortunately, very little functional evidence is available to support this hypothesis. In particular, it is not known whether the decrease in leaf NO_3^- reduction in the dark, as compared with in the light, results entirely from NR inactivation, or whether it can also be partly attributed to other putative mechanisms such as those described above (i.e. a shortage in the xylem NO_3^- supply to the leaf or limitation by NADH availability).

The work described in this paper was initiated to answer this question. This was possible because transgenic *N. plumbaginifolia* plants (Del transformants) in which both transcriptional and posttranscriptional regulation of NR are abolished have recently been obtained (Nussaume et al., 1995). These plants express a truncated *NIA* cDNA under the control of the 35S promoter, leading to the constitutive synthesis of a modified NR (ΔNR) with an N-terminal deletion of 56 aa. The ΔNR has lost sensitivity to the Mg^{2+}-ATP-dependent inactivation in vitro, and leaf levels of ΔNR mRNA, ΔNRP, and ΔNRA are no longer significantly affected by darkness in vivo (Nussaume et al., 1995). Our experiments were therefore aimed at determining whether the deregulation of ΔNR in the leaves of Del transformants prevents the usual decrease in leaf NO_3^- reduction, assayed using ^{15}N, under conditions in which photosynthesis is inhibited (i.e. when the native leaf NR is inactivated and degraded in the wild type).

To assess specifically the physiological role of the posttranscriptional regulation of NR, the Del transformants, lacking both transcriptional and posttranscriptional controls, were compared with both wild-type plants and the C1 transformant of *N. plumbaginifolia*, in which only the transcriptional level of regulation is suppressed because of the expression of a full-length *NIA*-cDNA under the control of the 35S promoter (Vincentz and Caboche, 1991).

MATERIALS AND METHODS

Plant Material

Genotypes Del 7 and Del 8 were obtained by transformation of the NR-deficient mutant E23 of *Nicotiana plumbaginifolia* with a Δ*NR* chimeric gene consisting of the *NIA2* cDNA of tobacco, with an internal deletion of 168 bp at the 5′ end, under the control of the cauliflower mosaic virus 35S promoter (Nussaume et al., 1995). Although missing 56 amino acids in its N-terminal part, the ΔNRP expressed in these transformants has been shown to be catalytically active, both in vitro and in vivo. Posttranscriptional regulation of ΔNR by light is apparently abolished in leaves of both Del genotypes (Nussaume et al., 1995). The use of the 35S promoter also results in the loss of transcriptional regulation of ΔNR.

Genotype C1 resulted from the transformation of the mutant E23 with full-length *NIA2* cDNA under the control of the 35S promoter (Vincentz and Caboche, 1991). As a result, transcriptional regulation of NR is abolished in this transformant. However, as in the wild type, the enzyme is still under posttranscriptional control in C1 and both leaf NRA and NRP levels are affected by darkness (Vincentz et al., 1993).

Plant Culture

Sterile seeds of *N. plumbaginifolia* were germinated in vitro on one-half-strength Murashige and Skoog medium. Seedlings with two to three emerged leaves were transferred to polypropylene boxes filled with a complete nutrient solution containing 0.1 mM KNO_3, 0.05 mM $Ca(NO_3)_2$, 0.2 mM KH_2PO_4, 0.2 mM $MgSO_4$, 10 μM KCl, 6 μM H_3BO_3, 1 μM $MnSO_4$, 0.2 μM $CuSO_4$, 0.02 μM $(NH_4)_6Mo_7O_{24}$, and 0.02 mM FeNa-EDTA, and the culture was continued in a growth chamber with an 8-h/16-h light/dark cycle, at 25°C/20°C, 350 μmol m^{-2} s^{-1} PPFD light intensity, and RH constant at 70%.

After approximately 1 week, the plants were transferred to 10-L plastic containers filled with a nutrient solution five times more concentrated than that used previously (e.g. containing 1 mM NO_3^-). After 2 additional weeks, the plants were selected for uniformity, and only 12 plants were left on the top of each container. The nutrient solution was replaced every 2 or 3 d and the day before the experiments. Plants were used for experiments at the rosette stage, when they had six to eight leaves.

Experimental Conditions

Unless otherwise stated, experiments were carried out in the same growth chamber and under the same light/dark regime as for culture of the plants.

For determination of net $^{15}NO_3^-$ uptake and reduction, the plants were transferred to fresh nutrient solution, pH 5.8, a few hours before the beginning of any measurement

and were left undisturbed until the end of the experiment. ^{15}N labeling was initiated at the dark/light or light/dark transition by adding 10 mM $K^{15}NO_3$ (99 atom % ^{15}N) to the nutrient solution containing $^{14}NO_3^-$ so that the final NO_3^- concentration was 1 mM. The exact ^{15}N enrichment of the $^{15}NO_3^-$ in the nutrient solution (around 20 atom % ^{15}N) was determined on a 20-mL aliquot sampled 15 min after ^{15}N addition. The plants were harvested after 8 h in light or 16 h in the dark, and the organs were weighed, dried, and ground for ^{15}N analysis in total N and NO_3^-, as described by Delhon et al. (1995a).

For determination of ^{15}N-NO_3^- incorporation into the amino-N pool of the leaves, the plants were transferred to fresh nutrient solution, pH 5.8, containing 1 mM $^{15}NO_3^-$ (99 atom % ^{15}N) for 8 h in the light or 16 h in the dark. At the end of the labeling period the leaves were weighed and rapidly stored in liquid N_2 prior to freeze-drying and grinding for ^{15}N analysis in amino acids.

The metabolic use of stored endogenous NO_3^- was measured in plants transferred for two successive light/dark cycles to a nutrient solution without NO_3^-, which was of similar composition to that used for growth except that KNO_3 and $Ca(NO_3)_2$ were replaced by K_2SO_4 and $CaCl_2$, respectively. A set of plants of each genotype was harvested at the end of each light or dark period, and both roots and shoots were weighed and oven-dried for 48 h at 70°C before assay of their NO_3^- content.

The $^{15}NO_3^-$ reduction in detached leaves was determined in two independent experiments, according to the procedure described by Delhon et al. (1996a). The detached leaves of each genotype were placed with their bases dipping in solutions containing $K^{15}NO_3$ at various concentrations (20 atom % ^{15}N) for 3 h in the light or dark, and then in deionized water for an additional hour. At the end of the 4-h experimental period the detached leaves were weighed and dried for 48 h at 70°C.

The dependence of leaf $^{15}NO_3^-$ reduction in the light on the in vivo rate of photosynthesis was investigated by modifying the CO_2 concentration in the atmosphere. Two independent experiments were performed. In both, detached leaves of the four genotypes were placed for 4 h on 10 mM $K^{15}NO_3$ (20 atom % ^{15}N) in a 240-L, airtight plexiglass chamber connected to a computerized device for controlling temperature, humidity, and CO_2 concentration in the atmosphere (Atelliance Instruments, Clapiers, France; see Delhon et al., 1996b, for details). In the first experiment five different treatments were applied: the CO_2 concentration in the atmosphere was held constant for the 4-h labeling period at 5, 50, 100, 200, or 400 $\mu L\ L^{-1}$.

In the second experiment in vivo rates of NO_3^- reduction and CO_2 fixation were simultaneously assayed with $^{15}NO_3^-/^{13}CO_2$ double labeling. Immediately after installation of the detached leaves on the $^{15}NO_3^-$ solution, the $^{12}CO_2$ initially present in the chamber was rapidly removed by switching on a carbosorb trap in the air circuit, and was replaced by $^{13}CO_2$ (11.59 atom % ^{13}C), which was added automatically from a pressurized bottle (GC015L10, Eurisotop, Saint-Aubin, France) until the nominal CO_2 concentration in the atmosphere was reached (the whole operation was completed in less than 15 min). The experimental protocol was the same as in the first experiment, except that the five treatments were at 5, 100, 200, 400 or 800 $\mu L\ L^{-1}$ $^{13}CO_2$. Samples of the atmosphere inside the chamber were periodically collected to assay the atom % ^{13}C of the $^{13}CO_2$ supplied to the leaves. After each treatment in both experiments, the detached leaves of all genotypes were rapidly weighed and dried for ^{15}N analysis in total N and NO_3^- or, when appropriate, for ^{13}C analysis in total C.

The effect of CO_2 on leaf in vitro NRA of the four genotypes was investigated by transferring intact plants for 30 min to an atmosphere in which the CO_2 concentration was either maintained at approximately 450 $\mu L\ L^{-1}$ or decreased to 0 $\mu L\ L^{-1}$.

Analytical Procedures

To determine endogenous NO_3^- reduction NO_3^- was extracted from the dried organs with 20 mL of 0.1 N HCl for 48 h at 4°C. For $^{15}NO_3^-$ analysis the extraction was carried out on 20 mg of freeze-dried, ground material with 4 mL of deionized water at 4°C for 48 h. After reduction to NO_2^- on a Cd column and the addition of sulfanilamide and *N*-(1-naphthyl)ethylene diamine dihydrochloride, NO_3^- concentrations in the extracts or in the solutions were assayed colorimetrically using a continuous-flow automated device. NO_2^- was extracted from fresh material with boiling water and was assayed as described previously (Robin et al., 1983).

The total ^{15}N and ^{13}C contents of the samples were measured using an on-line MS system (ANCA, Europa Scientific, Crewe, UK). The atom % ^{15}N of NO_3^- in the samples was determined according to the method of Prosser et al. (1993). The reduction of $^{15}NO_3^-$ in intact plants or detached leaves was calculated from the difference between total ^{15}N and $^{15}NO_3^-$ accumulated in the tissues.

Amino acids were extracted from freeze-dried, ground samples by mixing 100 mg of sample with 5 mL of 2% (w/v) sulfosalicylic acid in water at 4°C, followed by centrifugation at 17,500*g* for 15 min at the same temperature. The supernatants were adjusted to pH 2.0 and stored at −40°C until amino acid analysis. The amino acids were separated by ion-exchange chromatography (LC 5001 analyser, Biotronik, Maintel, Germany) using lithium citrate buffers and ninhydrin postcolumn derivatization. They were identified using a mixture of amino acids (standard PANB, Benson, Reno, NV) and quantified using a computer software program (version 2100, P.E. Nelson, Perkin-Elmer).

To measure the incorporation of ^{15}N into amino acids, 100 mg of freeze-dried, ground samples were extracted at 4°C in 10 mL of methanol:chloroform:water (12:5:3, v/v/v) for 60 min. The homogenate was centrifuged at 13,000*g* for 20 min. The pellet was extracted twice and the supernatants combined. The addition of 6 mL of chloroform and 4 mL of distilled water to the supernatants induced phase separation. After centrifugation at 800*g* for 5 min, the methanol:water fraction was collected and dried under a vacuum using a rotary evaporator, and then redissolved in 1 mL of distilled water. The resulting samples were then

applied to 5.0- × 0.5-cm Dowex-50 H^+ columns and washed with 5 mL of distilled water, and the amino acids and amides were eluted with 4 mL of 6 M NH_4OH. The amino acid fraction was lyophilized and redissolved in 1 mL of 50% methanol.

The purified extract (0.5 mL) was transferred directly to a silanized vial, dried under N_2, and derivatized with *N*-methyl-*N*-(tert-butyldimethylsilyl)-trifluoroacetamide (Pierce) in pyridine, as described by Rhodes et al. (1989). The atom % ^{15}N of each amino acid (including the proportion of Gln singly or doubly labeled) was then determined by GC-MS analysis with a quadrupole system (MD800, Fisons, Beverly, MA) according to the method of Cliquet and Stewart (1993).

In vitro NRA in leaves was assayed according to the method of Nussaume et al. (1995). The extracts were incubated for 10 min at 20°C in the presence of 10 mM $MgCl_2$ with or without 15 mM EDTA. The activation state of NR was calculated by dividing the NRA without EDTA by the NRA with EDTA.

RESULTS

Uptake and Reduction of Exogenous $^{15}NO_3^-$ in Light and Darkness

Biomass accumulation in the plants at the time of the experiments did not significantly differ between the four genotypes, but the root/shoot ratio was 10 to 15% lower in the transformants compared with the wild type (data not shown).

When expressed on a root fresh weight basis, the net uptake rate of $^{15}NO_3^-$ was similar in the four genotypes (Fig. 1A). Nevertheless, the lower root/shoot ratio of the transformants resulted in a total $^{15}NO_3^-$ uptake per plant that was slightly lower in the C1 and Del genotypes than in the wild type (data not shown). Net $^{15}NO_3^-$ uptake was apparently independent of illumination, since the mean rates calculated for the whole light or dark periods of the day/night cycle were similar (Fig. 1A). However, total reduction of the exogenous $^{15}NO_3^-$ taken up by the plant was markedly decreased by darkness in all genotypes (Fig. 1B). Neither the mean rate of reduction nor its diurnal changes was significantly modified in the three transformants compared with the wild type (Fig. 1B).

Regardless of the genotype, both total and reduced ^{15}N contents were approximately the same in the root and shoot at the end of the light period (Fig. 2, A and C), whereas the dark period produced a much lower ^{15}N incorporation in total and reduced N fractions in the shoot than in the root (Fig. 2, B and D). The amount of NO_2^- accumulated in the leaves at the end of the dark period (not taken into account in the reduced ^{15}N fraction) was negligible in all genotypes (data not shown).

Incorporation of $^{15}NO_3^-$-N into Leaf Amino Acids in the Light and the Dark

Although total amino acid content of the leaves was not drastically affected by the genotype (Fig. 3A), Gln levels were higher in both light and dark in the leaves of the two

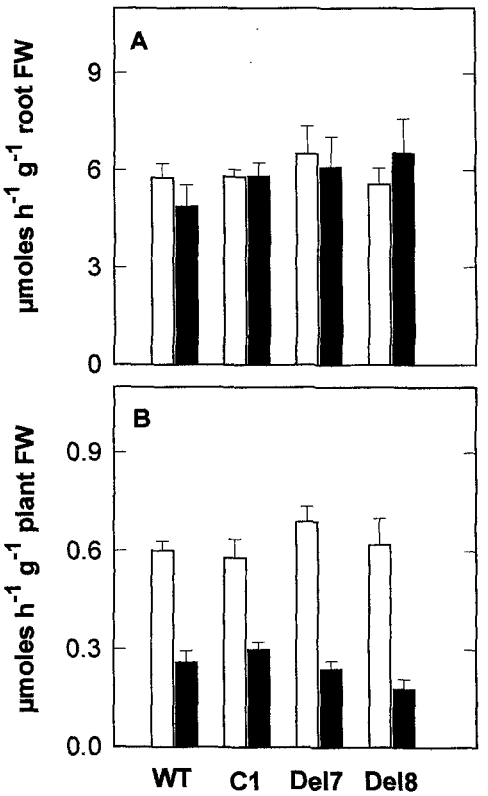

Figure 1. Mean rates of net $^{15}NO_3^-$ uptake (A) and reduction (B) in light or darkness. The *N. plumbaginifolia* plants of the four genotypes were supplied with 1 mM $^{15}NO_3^-$ (approximately 20 atom % ^{15}N) either for an 8-h light period (open bars) or for a 16-h dark period (closed bars). Net $^{15}NO_3^-$ uptake was calculated from total ^{15}N incorporation in the plant. Total $^{15}NO_3^-$ reduction was calculated as the difference between total ^{15}N and $^{15}NO_3^-$. The mean rates of net $^{15}NO_3^-$ uptake and reduction were calculated by dividing the total amount of total ^{15}N or reduced ^{15}N in the plant, respectively, by the number of hours in each treatment period. The results are the mean of five replicates ± SE. FW, Fresh weight; WT, wild type.

Del transformants compared with those of wild-type and C1 plants (Fig. 3B). The determination of ^{15}N incorporation into total amino acid or into Gln indicated that this was not due to increased $^{15}NO_3^-$ assimilation in the Del leaves (Fig. 3, C and D). The amounts of newly synthesized ^{15}N-Gln were generally very similar in the leaves of all four genotypes, and at least 50% lower in the dark than in the light (Fig. 3D). Therefore, the diurnal changes in the ^{15}N labeling of the amino-N pool reflected those observed for total $^{15}NO_3^-$ reduction in the whole plant (Fig. 1B).

Time Course of Endogenous NO_3^- Reduction during the Day/Night Cycle

To rule out the hypothesis that the above data concerning exogenous $^{15}NO_3^-$ utilization are not representative of total NO_3^- assimilation (since part of this is due to reduction of endogenous $^{14}NO_3^-$), an experiment was carried out to assay the diurnal changes in the reduction of previously stored NO_3^- (Fig. 4). The transfer to a N-deprived nutrient solution for 48 h resulted in a nearly 75% decrease

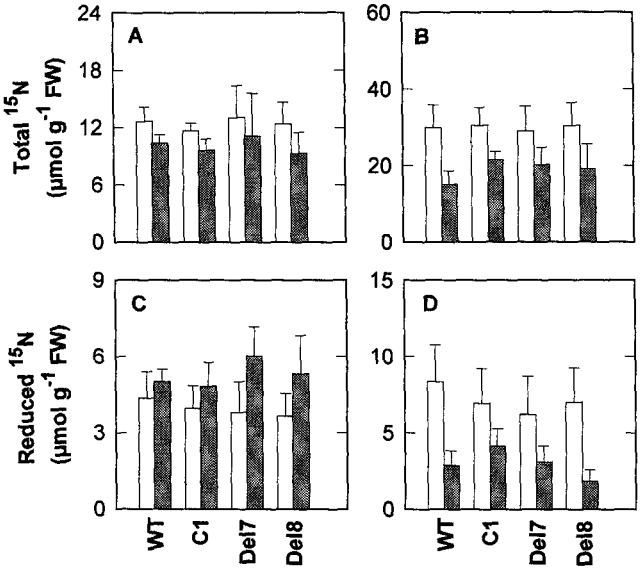

Figure 2. Incorporation of ^{15}N in root and shoot total N (A, B) and reduced N (C, D) fractions, at the end of an 8-h labeling period in the light (A, C) or a 16-h labeling period in the dark (B, D). Open bars, Root; shaded bars, shoot. Other experimental details are as in Figure 1. The results are the mean of five replicates ± SE. FW, Fresh weight; WT, wild type.

in the total NO_3^- content of the plants. As observed for externally supplied $^{15}NO_3^-$, endogenous NO_3^- was metabolized predominantly in the light in all genotypes. Depending on the genotype, the rates of NO_3^- disappearance ranged from 6 to 8 µmol h^{-1} $plant^{-1}$ and from 2 to 6 µmol h^{-1} $plant^{-1}$ during the first and second light periods,

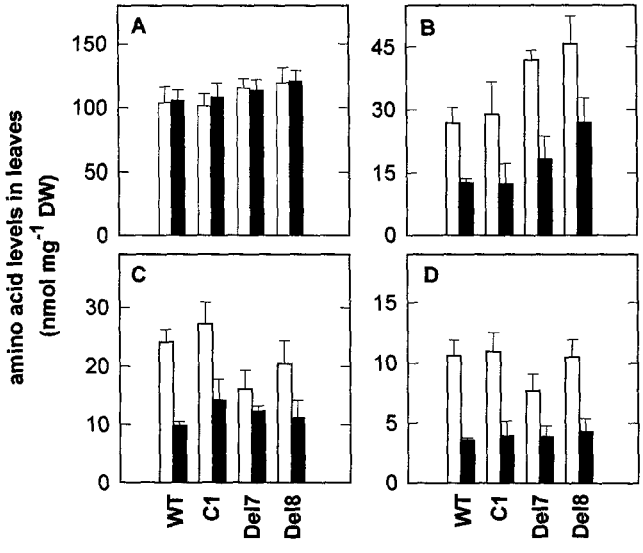

Figure 3. Total free amino acid (A) or Gln (B) contents of the *N. plumbaginifolia* leaves, and incorporation of ^{15}N-NO_3^- into the leaf total amino acid fraction (C) or into Gln (D). The leaves of the four genotypes were harvested after labeling of the plants with 1 mM $^{15}NO_3^-$ (99 atom % ^{15}N) either for an 8-h light period (open bars) or for a 16-h dark period (closed bars). The results are the mean of four replicates ± SE. DW, Dry weight; WT, wild type.

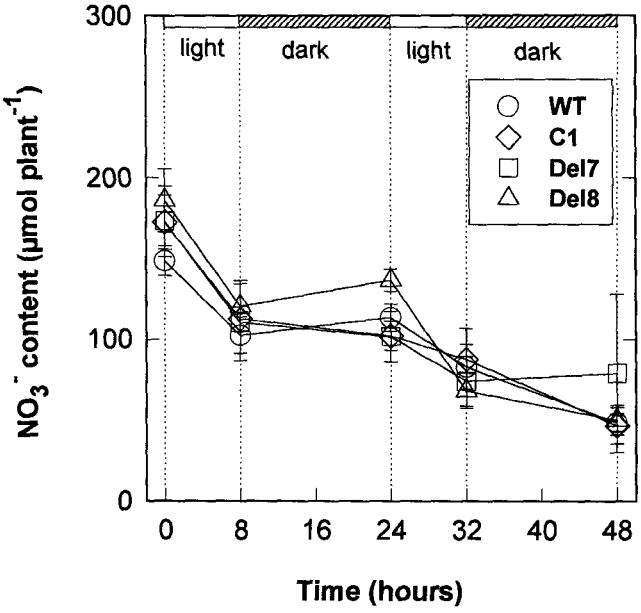

Figure 4. Time course of total NO_3^- content of *N. plumbaginifolia* plants after transfer to an N-deprived nutrient solution. At the onset of the light period the plants of all four genotypes were transferred for 48 h to a N-free nutrient solution in which KNO_3 and $Ca(NO_3)_2$ were replaced by K_2SO_4 and $CaCl_2$, respectively. A set of plants of each genotype was harvested at the end of each light or dark period. The results are the means of five replicates ± SE. WT, wild type.

respectively. The corresponding values for these rates during the first and second dark periods were 0 to 0.6 µmol h^{-1} $plant^{-1}$ and from 0 to 3.5 µmol h^{-1} $plant^{-1}$, respectively. Considering the 48-h experimental period, no significant difference in the decay of total NO_3^- content was observed between the three transformants and the wild type.

Reduction of $^{15}NO_3^-$ in Detached Leaves in Light and Dark

Because root NO_3^- reduction may be different in each genotype, the above results from whole-plant measurements of NO_3^- assimilation are not conclusive regarding the light dependence of this process in leaves. To specifically investigate this dependence, $^{15}NO_3^-$ reduction in detached leaves supplied with $^{15}NO_3^-$ through the xylem was determined either in the light or in the dark. Figure 5 presents the results of two independent experiments in which $^{15}NO_3^-$ reduction in Del7 or Del8 leaves was compared with that in C1 or wild-type leaves, respectively.

Under both light and dark conditions leaf $^{15}NO_3^-$ reduction was strongly dependent on exogenous $^{15}NO_3^-$ supply. In some instances $^{15}NO_3^-$ reduction in the dark at the highest level of $^{15}NO_3^-$ supply exceeded $^{15}NO_3^-$ reduction in the light at the lowest level of $^{15}NO_3^-$ supply (Fig. 5A). However, for a given xylem flux of $^{15}NO_3^-$, $^{15}NO_3^-$ reduction in the detached leaves of all genotypes was always much lower in the dark than in the light (Fig. 5).

Neither the abolition of posttranscriptional regulation of NR (comparison of Del and C1 genotypes, Fig. 5A) nor the

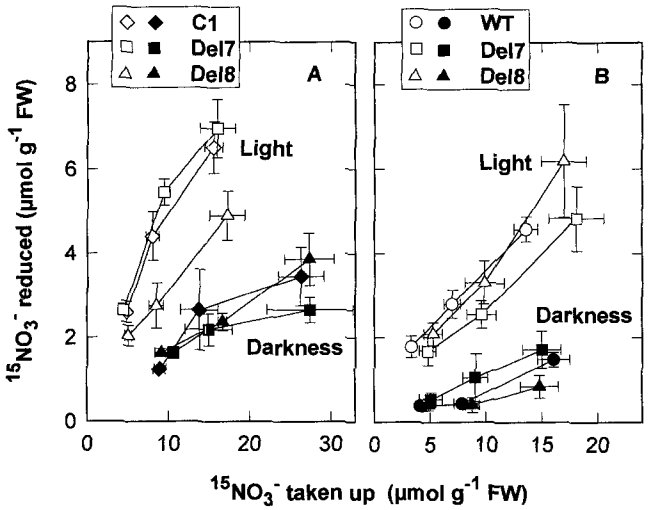

Figure 5. Reduction of $^{15}NO_3^-$ in detached *N. plumbaginifolia* leaves in the light or in the dark. The leaves were excised 4 h after the onset of light and transferred for 3 h to polypropylene vials containing 5, 10, or 20 mM $K^{15}NO_3$ (20 atom % ^{15}N) in the light, or 15, 30, or 60 mM $K^{15}NO_3$ (20 atom% ^{15}N) in the dark. After this treatment all leaves were put into deionized water for an additional hour under the same light conditions as for ^{15}N labeling. The amount of $^{15}NO_3^-$ taken up was calculated from the amount of total ^{15}N in the leaf. Leaf reduction of $^{15}NO_3^-$ was calculated from the difference between total ^{15}N and $^{15}NO_3^-$. The results shown in A and B are from two independent experiments and are the mean of six replicates. Horizontal and vertical bars indicate SE. FW, Fresh weight; WT, wild type.

abolition of both transcriptional and posttranscriptional regulations of NR (comparison of Del and wild-type genotypes, Fig. 5B) resulted in a modified effect of light on leaf $^{15}NO_3^-$ reduction.

Reduction of $^{15}NO_3^-$ in Illuminated Detached Leaves as Affected by $^{13}CO_2$ Concentration in the Atmosphere

Pretreatment of the plants for 30 min in a CO_2-free atmosphere did not affect the activation state of ΔNR in the leaves of the Del transformants, whereas it significantly decreased that of native NR in wild-type and C1 leaves (Fig. 6).

More detailed modifications of the CO_2 concentration in the atmosphere indicated that leaf $^{15}NO_3^-$ reduction in the light is highly dependent on the current photosynthate production (Fig. 7). In the detached leaves of all genotypes $^{15}NO_3^-$ reduction decreased when net $^{13}CO_2$ fixation decreased to values below 50 µmol g^{-1} leaf fresh weight. However, this decrease was consistently more pronounced in leaves of wild-type and C1 plants than in those of Del7 and Del8 plants. At a CO_2 concentration similar to that of standard air (400 µL L^{-1}), leaf $^{15}NO_3^-$ reduction was approximately the same in all genotypes, but decreased by more than 60% in wild-type and C1 plants in the absence of CO_2, whereas only a 30% decrease was observed in Del7 and Del8 plants (Fig. 7).

Figure 8 summarizes the results from two independent experiments and shows that lowering CO_2 concentration in the atmosphere resulted in a gradual stimulation of leaf $^{15}NO_3^-$ reduction in Del7 and Del8 plants compared with that in wild-type plants (ranging from 60–110% when CO_2 was nearly absent from the atmosphere). Similar results were obtained when comparing $^{15}NO_3^-$ reduction in Del7 and Del8 leaves with that in C1 leaves, with a corresponding stimulation ranging from 40 to 110% at 5 µL L^{-1} CO_2 in the atmosphere (Fig. 7 and other data not shown).

DISCUSSION

Our results indicate that the light/dark treatments applied to intact plants or to detached leaves led to changes in NO_3^- reduction that were almost identical for all four *N. plumbaginifolia* genotypes investigated (Figs. 1–5). Therefore, in the conditions of this study, transcriptional and posttranscriptional regulations of NR as affected in the C1 and Del transformants had little effect on the control of NO_3^- reduction by light. The absence of effect of constitutive expression of the *NIA* gene in the C1 transformant was expected, since in C1, as in the wild type, both leaf NRP and NRA have been shown to decrease in the dark (Vincentz et al., 1993).

The small in vivo impact of the abolition of posttranscriptional regulation of NR was much more surprising. In the leaves of the Del transformants, light/dark changes in ΔNRP and ΔNRA were suppressed (Nussaume et al., 1995). The reason for this is unknown, but it suggests that the N-terminal part of NR, which is missing 56 amino acids in the ΔNR protein, may play a role in the recognition of the enzyme by the inactivating 14-3-3 proteins or by specific proteases (Moorhead et al., 1996). In spite of the high activation state of the leaf ΔNR in the dark, neither exogenous $^{15}NO_3^-$ nor endogenous $^{14}NO_3^-$ reduction was stimulated in the Del transformants in the dark compared

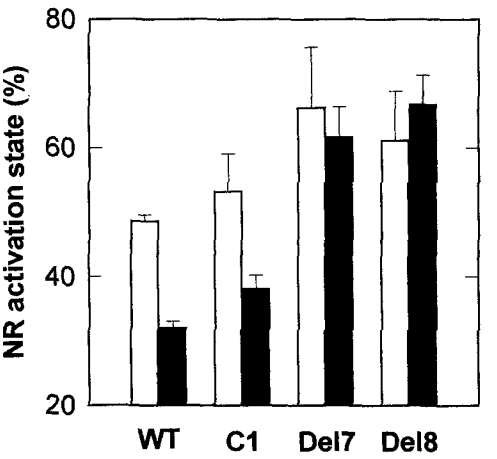

Figure 6. Effect of CO_2 removal from the atmosphere on the activation state of NR in *N. plumbaginifolia* leaves. The intact plants of the four genotypes were maintained for 30 min in an airtight chamber in which the CO_2 concentration in the atmosphere was either maintained at approximately 450 µL L^{-1} (open bars) or decreased to 0 µL L^{-1} (closed bars). The NR activation state was determined by comparison of –EDTA and +EDTA (15 mM) assays. The results are the mean of three replicates ± SE. WT, Wild type.

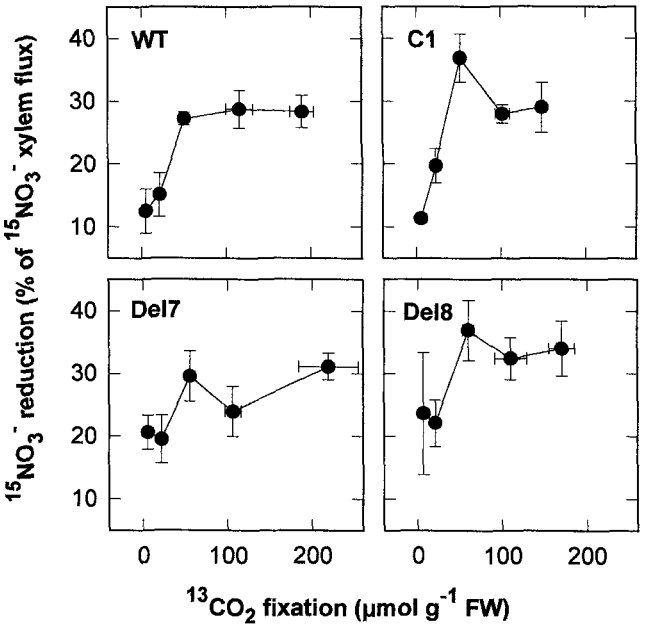

Figure 7. Dependence of $^{15}NO_3^-$ reduction on $^{13}CO_2$ fixation in illuminated detached leaves of *N. plumbaginifolia*. The leaves of the four genotypes were excised either 1 h (5, 100, or 800 μL L^{-1} CO_2) or 4 h (200 or 400 μL L^{-1} CO_2) after the onset of light, and transferred to polypropylene vials containing 10 mM $K^{15}NO_3$ (20 atom % ^{15}N) for 4 h in the light. After the removal of $^{12}CO_2$ from the atmosphere, $^{13}CO_2$ (11.6 atom% ^{13}C) was added to obtain the desired concentration (5, 100, 200, 400, or 800 μL L^{-1}). Net fixation of $^{13}CO_2$ was determined as the amount of total ^{13}C in the leaf. Xylem flux of $^{15}NO_3^-$ was calculated as the amount of total ^{15}N in the leaf and did not differ significantly between the genotypes. Leaf reduction of $^{15}NO_3^-$ was calculated as the difference between total ^{15}N and $^{15}NO_3^-$, and was normalized by expressing the results as the percentage reduction of the $^{15}NO_3^-$ taken up by the leaf. The results are the mean of six replicates. Horizontal and vertical bars indicate SE. FW, Fresh weight; WT, wild type.

with that observed in C1 or wild-type plants (Figs. 1, 2, 4, and 5).

Furthermore, we show here that the high Gln content in the Del leaves reported by Nussaume et al. (1995) is not indicative of increased NO_3^- reduction in the dark, since it is observed in both the light and the dark, and is not related to recent de novo synthesis of this amino acid (Fig. 3). Although not seen in the experiment shown in Figure 3, an increase in leaf Gln accumulation compared with wild type has also been noticed in the C1 transformant (Quilleré et al., 1994), suggesting that it may not be specifically related to the abolition of posttranscriptional regulation of NR, but more likely to constitutive expression/overexpression of the *NIA* gene. Overexpression of NR may explain why leaf $^{15}NO_3^-$ reduction in the C1 and Del transformants sometimes exceeded that in the wild type in the light as well as in the dark (compare Fig. 5, A and B).

A critical role of posttranslational inactivation of NR for limiting in vivo NO_3^- reduction has not only been proposed for light/dark transition, but also for various conditions in which photosynthesis is affected in the light (e.g. CO_2 deprivation or stomatal closure in response to drought; see Kaiser and Huber, 1994a). Indeed, short-term inhibition of photosynthesis in the light due to the removal of CO_2 from the atmosphere resulted in both a decreased leaf NR activation state and a decreased leaf $^{15}NO_3^-$ reduction in both wild-type and C1 *N. plumbaginifolia* (Figs. 6 and 7).

Under these conditions two observations support the hypothesis that the decrease in leaf NO_3^- reduction in wild-type and C1 genotypes is partly due to NR inactivation. First, the hyperbolic dependence of leaf $^{15}NO_3^-$ reduction on in vivo rates of CO_2 fixation (Fig. 7) closely resembles that previously described for leaf NRA (De Cires et al., 1993). Second, the abolition of posttranscriptional regulation of ΔNR in the Del transformants, which resulted in the expected loss of ΔNR inactivation in the absence of CO_2 (Fig. 6), was associated with a modified regulation of in vivo $^{15}NO_3^-$ reduction in the illuminated leaves; the dependence of $^{15}NO_3^-$ reduction upon continuous photosynthate production was strongly attenuated in Del leaves compared with C1 and wild-type leaves (Figs. 7 and 8).

Collectively, our results lead to the conclusion that deregulation of NR at the posttranscriptional level in the Del transformants had functional consequences when photosynthesis was prevented by CO_2 deprivation in the light but not when it was prevented by darkness. This suggests that posttranscriptional regulation of NR is responsible, at least partially, for the short-term coordination between NO_3^- and CO_2 assimilation in the illuminated leaves, but not for the light/dark changes in NO_3^- assimilation. This observation is consistent with the idea that modulation of NRA is not the only mechanism involved in the control of leaf NO_3^- reduction in the dark.

As mentioned previously, additional regulatory factors may include limitation by reducing power (Abrol et al., 1983) or shortage of NO_3^- translocation resulting from

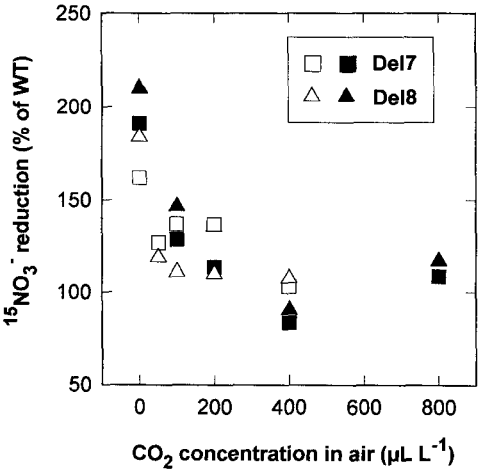

Figure 8. Stimulation of leaf $^{15}NO_3^-$ reduction in the two Del transformants compared with that in the wild type, in response to the decrease in CO_2 concentration in the atmosphere. Leaf $^{15}NO_3^-$ reductions in the two Del transformants are expressed as a percentage of that in the wild type. The results come from two independent experiments (closed symbols for experiment shown in Fig. 5, open symbols for the other experiment). WT, Wild type.

reduced transpiration (Rufty et al., 1984; Delhon et al., 1995a, 1995b). Although leaf $^{15}NO_3^-$ reduction in the dark is strongly dependent on xylem supply of $^{15}NO_3^-$ (Fig. 5), two observations indicate that restricted NO_3^- translocation cannot be the main cause of the dark inhibition of leaf NO_3^- reduction in *N. plumbaginifolia*. First, darkness markedly diminished $^{15}NO_3^-$ reduction in detached leaves, even when the $^{15}NO_3^-$ xylem flux was set at the same value as in the light (Fig. 5). Second, NO_3^- accumulation in the leaves of intact plants increased during the night (data not shown), which reveals that leaf NO_3^- reduction was affected to a greater extent by darkness than was NO_3^- translocation. As a consequence, we hypothesize that leaf NO_3^- reduction in the dark is limited by both NR activity and the availability of reducing power. Thus, the strength of the energetic limitation may prevent any significant effect of the abolition of NR inactivation alone, as was seen in our experiments (Figs. 1–5).

Unlike dark conditions, the light and CO_2-deprivation conditions resulted in increased $^{15}NO_3^-$ reduction in Del leaves compared with C1 or wild-type leaves (Figs. 7 and 8), suggesting that the limitation by reducing power was alleviated, and that the activation state of NR exerted a much more powerful control of in vivo NO_3^- reduction. The reason for this may be related to the fact that under CO_2 deprivation in the light (i.e. under photorespiratory conditions), the excess reducing equivalents synthesized in the chloroplast may be exported to the cytoplasm via malate/oxaloacetate or triose-P/PGA shuttles (Heldt and Flügge, 1987), where they may generate enough NADH to saturate NR activity. However, the finding that the dependence of leaf $^{15}NO_3^-$ reduction on in vivo CO_2 fixation was not completely suppressed in the Del transformants (Fig. 7) indicates that unknown factors independent of NRA are still involved in the control of NO_3^- reduction in the light.

Although the above energetic limitation hypothesis fits well with our observations, alternative explanations may be proposed to account for the low impact of the Δ mutation of NR on the NO_3^- reduction rate in the dark. The ΔNR was shown to be unstable in vitro, and most of the biochemical assays done on this enzyme were performed after 30 min of dark treatment only (Nussaume et al., 1995), which is fairly different from the time intervals used in our study to investigate NO_3^- reduction in the dark (4–16 h). Thus, one possibility for reconciling biochemical and physiological data is to imagine that in vivo, the ΔNR is less stable in the dark than in the light, or that its inactivation occurs at a much slower rate than the native NR, and is consequently not seen after 30 min in the dark.

However, these hypotheses are in contradiction with the report that ΔNRA in the leaves of the Del transformants is still high after an extended (70-h) dark period (Nussaume et al., 1995). Another complication is the recent finding that, independently from phosphorylation, leaf NR is also subject to inactivation by slow conformational change (hysteresis) in response to light/dark transition (Lillo, 1994b; Huber and Huber, 1995). The hysteretic behavior of NR can only be seen using short (1-min) measurements of NRA (Lillo, 1994b). Thus, it cannot be concluded from the 15-min measurements performed by Nussaume et al. (1995) whether the ΔNR in the Del transformants is sensitive or not to regulation by hysteresis. As a consequence, we cannot rule out the hypothesis that, although not affected by the phosphorylation control, the ΔNR was inactivated in the dark, resulting in the observed low NO_3^- reduction rate assayed in the Del transformants. To our knowledge, the hysteretic behavior of leaf NR in response to inhibition of photosynthesis in the light has not been investigated, precluding any speculation on the role of this regulatory mechanism during our experiments of CO_2 deprivation.

Much additional work is still required at the molecular/biochemical level to understand how the known mechanisms of NR regulation act to tune the activity of the enzyme in relation to the C status of the plant, and how these mechanisms are affected in the Del transformants to yield a light- and CO_2-independent level of measurable ΔNRA. From a physiological viewpoint, the most striking conclusion of our work is that the apparent loss of ΔNR regulation in the leaves of the Del transformants had a limited in vivo impact. This is in agreement with the observations that the abolition of transcriptional and posttranscriptional regulations of NR have little consequence on plant growth (Vincentz and Caboche, 1991; Quilleré et al., 1994; Nussaume et al., 1995).

With the exception of a higher Gln accumulation in the leaves already observed in the C1 transformant (Quilleré et al., 1994), the only phenotype associated with ΔNR deregulation at the posttranscriptional level is a partial loss of the short-term coupling between photosynthesis and leaf NO_3^- reduction in the light (Figs. 6 and 7). However, unless CO_2 fixation is completely inhibited, the effect on the absolute amount of NO_3^- assimilated remains modest. Future research will need to confirm the generality of this phenotype and to investigate its significance in the long term, especially with regard to the regulation of amino acid synthesis in response to stomatal closure, increased photorespiration, or variations in light intensity.

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